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JOURNAL OF MEMBRANE BIOLOGY, (1999 Mar 1) 168 (1) 77-89.

Proc. Natl. Acad. Sci. U. S. A. (1993), 90(10), 4591-5

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Thank-You!

*Karen A. Lacourciere Ph.D.*

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(703) 308-7523

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## Endothelial gene regulation by biomechanical forces

Nitzan Resnick<sup>1</sup>, Sumpio E. Bauer<sup>2</sup>, Wei Du<sup>2</sup> and Michael A. Gimbrone Jr.<sup>1</sup>  
<sup>1</sup>Vascular Research Division, Department of Pathology, Brigham and Women's Hospital, Boston, Massachusetts;  
and <sup>2</sup>Department of Vascular Surgery, Yale University, New Haven, Connecticut, USA

**Abstract.** Hemodynamic forces generated by the pulsatile flow of blood through the circulatory system have been shown to influence the structure and function of vascular endothelium. Several groups, using in vitro systems, have demonstrated that defined biomechanical forces, including wall shear stress and cyclic strain, can modulate endothelial gene expression. In particular, our group has demonstrated that PDGF B-chain gene transcription is induced by exposure of cultured endothelial cells to a physiologic level of laminar shear stress. We have defined a region within the PDGF B-chain promoter that is responsible for this shear-induced gene expression, and have called this the "Shear Stress Response Element" (SSRE). This promoter element binds nuclear proteins extracted from shear-stressed endothelial cells. A core sequence (GAGACC) within the SSRE is also present in the promoters of several other endothelial genes that are responsive to shear stress. This core sequence was shown by electromobility shift assays to be a nuclear-protein binding site. Moreover, hybrid promoters containing the SSRE sequence (as present in the PDGF B-chain promoter) were inducible by shear stress when transfected into bovine aortic endothelial cells, thus confirming that this element is both necessary and sufficient for gene induction by laminar shear stress. We have recently extended these studies to another physiologically relevant hemodynamic force, cyclic strain, induced by biaxial stretching. Interestingly, the SSRE binds to nuclear proteins extracted from endothelial cells, but not from smooth muscle cells, exposed to the same level of cyclic strain (10% average strain, 60 cycles/min). These results suggest that different biomechanical forces may act on the endothelium through a common, but cell-type-specific, mechanism to activate gene transcription.

### Introduction

Vascular endothelial cells in direct contact with flowing blood bear the frictional forces imparted by this fluid, and also transmit wall tension derived from systolic-diastolic pressure changes acting perpendicular to the vessel lining. In vivo, these hemodynamic forces appear to affect endothelial structure and function, and have been implicated in the changes in macromolecular permeability, lipoprotein accumulation and cell damage that are associated with branch points and bifurcations [1-4]. In vitro systems have been developed to study the mechanisms of these physical effects on endothelial cells [5,6], and have confirmed the direct cellular actions of both fluid shear stresses and cyclic strains. More recently, a number of these hemodynamic effects have been shown to involve changes in endothelial gene expression [7,8]. Using a well-characterized fluid mechanical system, the cone and plate apparatus developed in collaboration with Prof. C.F. Dewey (Fluid Mechanics Laboratory, Massachusetts Institute of Technology, Cambridge, MA), we have studied the regulation of several pathophysiologically relevant genes after exposure of cultured endothelial monolayers (bovine, human) to defined fluid shear stresses. Our experimental approach was: (a) to identify candidate genes that are

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transcriptionally regulated by laminar shear stress in cultured endothelial cells; (b) to examine the 5' flanking regions of these genes for "shear-stress response elements"; and (c) to extend these studies to other types of biomechanical forces, e.g., cyclic strain.

Our initial studies have focused on the human PDGF B-chain gene [9]. This gene is transcriptionally upregulated as early as 30 min after the exposure of cultured endothelial cells to a physiological level of laminar shear stress (10 dynes/cm<sup>2</sup>). We identified a 50-bp region within the PDGF B-promoter responsible for the inducibility of this gene by shear stress, using deletion constructs of the PDGF B promoter. Using electromobility shift assays (EMSA), a 12-bp shear-stress response element (SSRE) was further defined. This element binds nuclear proteins from endothelial cells exposed to laminar shear stress, but does not encode a consensus binding site for any of the known transcription factors. Database analyses revealed that several promoters of endothelial genes responsive to shear stress contain the same 6-bp core sequence (GAGACC) present in the SSRE in the PDGF B gene. Further studies in our group [10] have demonstrated a selective pattern of induction of endothelial-leukocyte adhesion molecules (ICAM-1, but not VCAM-1 or E-selectin) that is correlated with the presence or absence of this SSRE core sequence in their promoters.

Recently, it has been demonstrated that cyclic strain can also regulate endothelial-cell gene expression in vitro, and that several endothelial genes are responsive to both cyclic strain and shear stress [11]. Here, we will review data demonstrating that: (a) the SSRE core sequence is the binding site for nuclear proteins induced by laminar fluid shear stress; (b) SSRE is both necessary and sufficient for gene induction by shear stress in endothelial cells; and (c) nuclear proteins extracted from endothelial, but not from smooth muscle cells, subjected to cyclic strain bind to the SSRE. These results suggest a cell-type-specific mechanism for endothelial gene induction by various types of hemodynamic forces.

## Materials and Methods

### *Shear stress apparatus*

The design of the cone and plate flow apparatus has been described [5]. In all the following experiments endothelial cells (bovine aortic endothelial cells (BAEC) or human umbilical vein endothelial cells (HUVEC)) were grown under standard culture conditions [5,9] on polystyrene tissue-culture coated coverslips, and were exposed to a physiologic level of laminar shear stress (10 dynes/cm<sup>2</sup>), or incubated under static (no-flow) conditions.

### *Cyclic strain*

BAEC or HUVEC were exposed to biaxial stretching on a Flexercell system (FLEXER-CELL Inc. Corp., McKeesport PA), as described elsewhere [6]. Both cell types were exposed to the same 10% average strain (60 cycles/min) for various time intervals.

### *Nuclear extracts and EMSA*

Nuclei were extracted from cells exposed to shear stress, cyclic strain, or static (control) conditions, as described previously [9]. Nuclear proteins were incubated with <sup>32</sup>P-labeled oligonucleotide probes (30 mer) at room temperature under low-salt conditions [9], and then analyzed on 4% acrylamide gels.

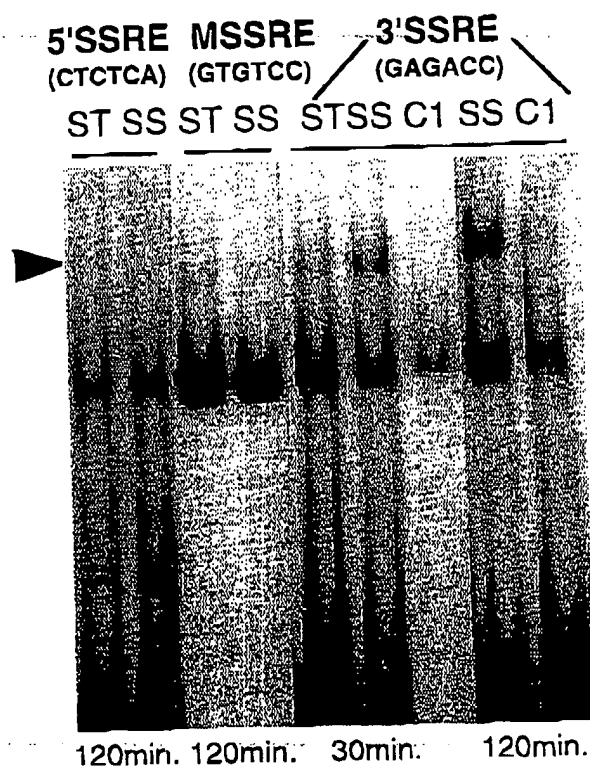
### Hybrid promoters

SSRE hybrid promoters were constructed using an enhancerless SV40/CAT vector (Promega, Madison, WI). The various constructs were transfected into BAEC by the modified calcium phosphate method [9]. Transfected cells were either grown under static conditions or exposed to flow, and CAT activity was measured and normalized to transfection efficiency.

### Results

#### *SSRE core sequence is a binding site for shear-stress-inducible nuclear proteins in endothelial cells*

Nuclei were extracted from BAEC exposed to laminar shear stress (10 dynes/cm<sup>2</sup>) or incubated under static conditions for 1 h. Nuclear protein binding was assayed with a set of oligonucleotide probes encoding various permutations of the SSRE. An inducible DNA-protein complex was consistently observed (Fig. 1) with the probe carrying the 3' part of the SSRE (GAGACC), but not with the 5' part (CTCTCA) or with a mutated SSRE (CTCTCAGTGTCC). This inducible complex was demonstrable with nuclear extracts from cells exposed to shear for only 15 min, and was still evident after 4 h (data not shown).



**Fig. 1.** Characterization of the DNA binding site within the SSRE: Nuclear extracts derived from BAEC incubated under static conditions (ST), or subjected to laminar shear stress (10 dynes/cm<sup>2</sup>) (SS) were incubated with three SSRE-derived probes (30 mer each). Binding and EMSA conditions are described elsewhere [9]. The SSRE probes included the 5'SSRE (noncore) (CTCTCA), a mutated SSRE (MSSRE)(CTCTCAGTGTCC) and the SSRE core sequence (3'SSRE)(GAGACC). The latter probe was incubated with nuclei extracted from cells exposed to shear stress for either 30 min (left) or 120 min (right). Specificity of formation of complex (arrow) was tested by the addition of unlabeled identical probe (C1).

### *SSRE is sufficient for gene induction by laminar shear stress*

As revealed by database analyses, several genes responsive to shear stress contained the SSRE core sequence (GAGACC), or its complementary sequence (GGTCTC), in their promoters [9]. To test the role of the SSRE in conferring shear responsiveness directly, we constructed a vector containing a SV40 enhancerless promoter and the CAT reporter gene, which was itself uninducible by shear stress, and then added the SSRE or its complementary sequence. Specifically, the following hybrid reporter gene constructs were made: (a) the SSRE (CTCTCAGAGACC) in the context of flanking sequences from the human PDGF-B promoter (SV-SSRE/CAT); (b) the complementary sequence (alternate strand) of the SSRE (CTCTCAGGTCTC) [SV-(C)SSRE/CAT]; and (c) the 5' (nonbinding) part of the SSRE (CTCTCA) [SV-(5')SSRE/CAT]. Each oligonucleotide was coupled to the SV/CAT construct in the *ECO* RI-*Bgl* II site. BAEC transfected with these constructs were exposed to shear stress for 2 h, or incubated under static conditions. As seen in Table 1, the addition of the SSRE or its complementary sequence, but not the 5' part of the SSRE, converted the SV/CAT-uninducible backbone vector into a fluid shear-stress-responsive one.

### *Nuclear proteins from endothelial cells exposed to cyclic strain bind to the SSRE*

To test whether similar mechanisms are involved in gene induction by both shear stress and cyclic strain, we exposed BAEC or HUVEC to 10% average biaxial cyclic stretching (60 cycles/min) for various time intervals. Nuclear proteins were then extracted and tested for their ability to bind the SSRE probe. As can be seen in Fig. 2, nuclear proteins from HUVEC (as well as from BAEC, data not shown) formed a specific inducible complex with the SSRE probe, which was strongly evident at 30 min but rapidly declined to below control (unstretched) levels by 2 h. This kinetic profile thus differs from that obtained with nuclei of endothelial cells exposed to fluid shear stress in which a positive gel-shift was still evident at 4 h. Similar nuclear proteins/SSRE complexes did not form in cultured human aortic smooth muscle cells exposed to cyclic strain for various time intervals (data not shown).

*Table 1. Hybrid promoters containing the SSRE core sequence (GAGACC) or its complementary sequence (GGTCTC) are shear-stress responsive*

Construct	CAT activity		Fold induction
	Static	Shear	
CMV/CAT	275	261	1
SV/CAT	14	14	1
SV-SSRE/CAT	9	34	3.6
SV-(C)SSRE/CAT	11	40	3.8
SV-(5')SSRE/CAT	12	15	1.3

BAEC were transfected with various reporter genes containing: the cytomegalovirus (CMV) promoter, the SV40 enhancerless promoter, the SV40 promoter coupled to the SSRE taken from the PDGF B promoter (SV-SSRE/CAT), the SV40 promoter coupled to the SSRE complementary sequence (SV-(C)SSRE/CAT), and the SV40 promoter coupled to the 5' portion (non-core) of the SSRE (SV-(5')SSRE/CAT). The transfected cultures were either subjected to shear stress (10 dynes/cm<sup>2</sup>, 2 h) or incubated under static conditions. CAT activity was normalized to the efficiency of transfection.

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N 0 0.5 1 2 4 8 24 CM

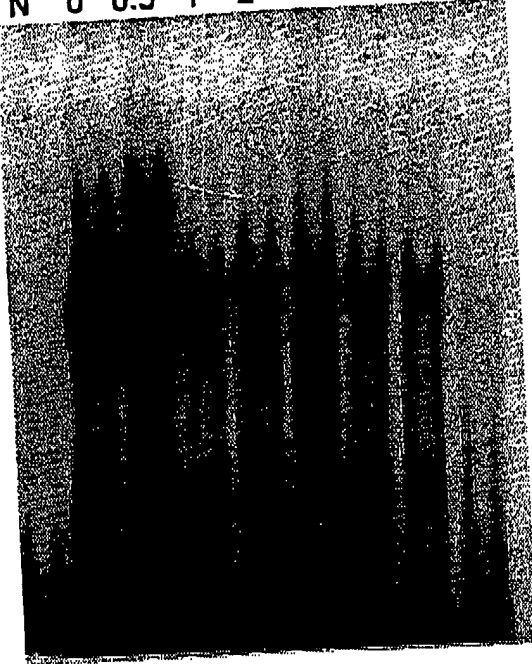


Fig. 2. Endothelial cells exposed to cyclic strain contain nuclear proteins that bind to the SSRE. HUVEC were exposed for various time intervals (0.5–24 h) to cyclic stretching (10% average strain, 60 cycles/min) in a FLEXERCELL system (FLEXERCELL Inc. Corp., McKeesport, PA) and their nuclei were extracted and incubated under conditions previously described [9] with a 30-mer PDGF B SSRE probe (containing the sequence CTCTCAGAGACC). A specific complex (arrow) was strongly induced 30 min after the exposure of the cells to cyclic strain but then declined to below control (unstretched) levels. N = no nuclear proteins, CM = competition with a 50-fold excess of a identical unlabeled probe.

## Discussion

Hemodynamic forces acting on the vascular endothelium play an important role in the structural remodeling of the vasculature and in the development of atherosclerotic lesions [1–4,12,13]. Although the effects of these forces on vessel wall components have been studied both in vitro and in vivo, the molecular mechanisms mediating these changes are far from understood. In the studies summarized here, we have focused on the human PDGF B-chain gene, as a model, and have examined the molecular mechanisms regulating its expression at the level of transcription. These studies have led to the identification and characterization of a “shear-stress response element, SSRE”, a cis-acting transcriptional regulatory element, which appears to be necessary for induction of this gene by physiological levels of shear stress. The insertion of this element into a minimal promoter construct generates a “hybrid promoter” that is shear-responsive and is sufficient to mediate induction of a reporter gene transfected into cultured endothelial cells. Although this SSRE does not encode a consensus binding site for known transcription factors, it does form specific complexes with nuclear proteins extracted from endothelial cells stimulated by exposure to physiological levels of laminar shear stress. The nature of these putative transcriptional factors that are activated by shear stress and interact with the SSRE in vascular endothelium is the subject of ongoing investigation.

In preliminary studies described here, we have examined the potential role of this molecular system in mediating gene regulation in response to a second type of hemodynamically generated force, cyclic strain. Unlike shear stress, which is a frictional

force imparted selectively to the endothelial lining, cyclic strain results from the stretching of the vessel wall induced by arterial pulsatile flow and acts on both endothelium and smooth muscle. Interestingly, cyclic stretching of endothelial cells, but not smooth muscle cells, upregulates SSRE-nuclear protein binding, after only 30 min. These findings suggest that the SSRE may mediate endothelial responses to different types of biomechanical forces, and that this mechanism may be specific to cell type. Further studies are required to elucidate the biomechanical transduction and second-messenger coupling cascades that are activated in endothelial cells by these hemodynamic forces.

In conclusion, the discovery of a "shear stress response element (SSRE)" in the promoter of several endothelial genes provides a potential genetic regulatory mechanism to explain various biomechanically induced changes in endothelial function. Consideration of this mechanism, as well as other effector systems in the vessel wall, leads to a working concept of the endothelial cell as an important transducer and integrator of the local pathophysiologic milieu.

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